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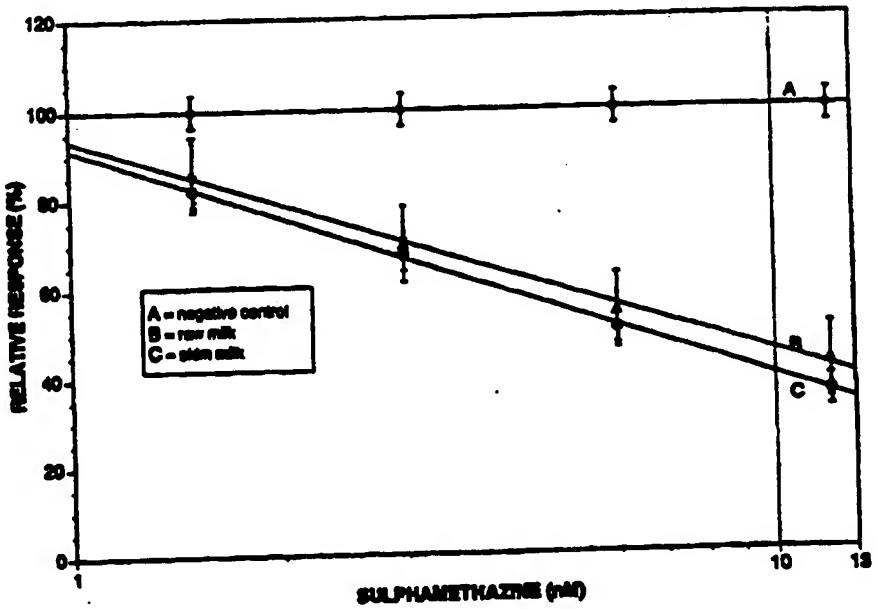

(57) Abstract

A method of determining the presence and/or amount of an analyte species in a raw milk sample, wherein the raw milk sample, without any preparation thereof for removal of milk constituents, is contacted with an optical sensor surface and the presence and/or amount of said analyte species is determined by measuring a change in refractive index at the surface caused by a specific interaction at the surface related to the presence of said species in the raw milk sample.

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MILK ASSAY

The present invention relates to a biosensor based assay method, and, more particularly, to a method for determining the presence and/or amount of an analyte species present in milk.

Before milk is used for consumption or processing in dairies, it is subjected to a number of quality tests, including the measurement of levels of various analyte species. One type of such analyte species is residues of veterinary antibiotics and chemotherapeutics. Milk samples are usually taken by the milk tank lorry driver, both at the milk producing farm and on the tank lorry. For practical reasons, the sample volumes taken are limited. For a particular analyte assay to fit into the control system, the assay must therefore require only a small sample volume. Thus, at present, a suspected milk sample can as a rule only be analyzed for a small number of antibiotics due to the limited sample volume. Many chromatographic methods, such as HPLC, are based on the concentration of a large sample volume for a sufficient sensitivity to be obtained and are therefore usually excluded from the current milk control systems.

Chromatographic techniques also have the limitations of the requirement of high technical expertise and lengthy and cumbersome sample preparation. For HPLC, for example, extraction procedures are critical for the generation of characteristic peaks and to avoid interference from co-eluting material.

Lengthy and cumbersome sample preparation is also required in the enzyme and radioimmunoassay techniques used today for the analyses of analyte species in milk. The necessary enzyme or isotope labelling of reagents is, of course, also a disadvantage, both from the viewpoint of sample handling and preparation of enzyme conjugates.

Microbiological assays, like the acidification control used today for detecting antibiotics, are slow (requiring long incubation steps), and not always reliable.

Microbiological assays can, of course, not be used on milk samples to which a preservative, such as e.g. bronopol, has been added in order to prevent growth of microorganisms during storage and transport of the milk sample to the laboratory. Such preservation is used for milk samples collected for a certain type of analysis, e.g. fat, protein and lactose.

The use of biosensors for the determination of species in milk has been described generally in several publications. For example, WO 92/16838 proposes the use of an optical immunosensor based on the surface plasmon resonance (SPR) principle for label-free detection of steroid hormones in various types of samples, including milk. The performance of such a milk assay, including sample preparation, is, however, not described in any detail and no examples demonstrating such an assay are presented.

EP-A2-0389446 discloses an optical measuring device for measuring the protein contents of milk by determining the refractive index of a drop of a milk sample on the surface of a refracting element.

The present invention aims to provide a method for determining the amount of an analyte species in milk which method requires a minimum sample volume and sample processing to prepare the sample for the assay, while simultaneously being quick and simple to operate and providing for sensitive and accurate measurements.

In accordance with the present invention, it has been found that by using an optical sensor surface, such as one of the above-mentioned surface plasmon resonance principle based type, the assay may be performed with high sensitivity directly on a raw milk (fresh milk) sample without subjecting the sample to any preparative steps, such as removal of fats etc. This is highly surprising as it would be expected that the relatively complex milk matrix medium would interfere too much with the analytical interactions. It has also surprisingly been found that the

addition of preservatives to the sample does not interfere with the analyses of at least some analyte species.

Accordingly, the present invention provides a method for determining the presence and/or amount of an analyte species in a raw milk sample, which method is characterized by contacting the raw milk sample, without any preparation thereof for removal of milk constituents, with an optical sensor surface and determining the presence and/or amount of the species by measuring a change in refractive index at the surface caused by a specific binding interaction at the surface related to the presence of the species in the raw milk sample.

The method requires only a small sample volume, no sample preparation and is easy to carry out. The method is also apt to automation and may thereby permit screening and/or quantification analyses of a great number of samples with a minimal effort.

Advantageously, the contacting of the sample with the surface is performed by passing the sample over the surface utilizing a liquid flow system.

It is readily understood that the use of apparatus permitting the monitoring of a plurality of (at least two) samples or a plurality of (at least two) analyte species with respective sensing surfaces will further increase the basic advantage of the invention of not having to process the raw milk samples prior to their use in the assay.

In one aspect of the invention, the analyte species or an analyte species analogue is bound to the optical sensor surface. Preferably, the optical surface has an organic polymeric layer, and binding is effected covalently and may take place through a linker molecule. In this aspect, the analysis takes the form of an inhibition assay in which an antibody against the analyte species is added to the sample. The change in refractive index at the optical sensor surface provides a measure of the amount of the free (i.e. unbound) antibody in the sample from which the concentration of the analyte species can be calculated.

In another aspect of the invention, a receptor, such as an anti-analyte species antibody, is bound to the optical sensor surface. The amount of analyte species in the sample may then be determined either directly by detecting binding of the analyte species to the receptor, or in a competitive assay by adding to the sample before it is passed over the surface, a substance which also binds to, and competes for, the binding site(s) of the receptor but which produces a greater change in refractive index on binding to the surface. Typically, in the competitive assay, the added substance will consist of the analyte species bound to a larger molecule or particle.

In the case of a large analyte molecule capable of exhibiting two or more receptor binding sites, the binding of the analyte species to the optical sensor surface may be detected in a sandwhich assay by a secondary reagent, such as an antibody, which binds to the bound analyte species. If desired, further specificity may be obtained by detecting binding of this secondary reagent by a tertiary reagent which binds to the secondary reagent.

Binding of the receptor to the surface may be carried out in conventional ways well-known to those skilled in the art. If, for example, the optical surface has a polymeric organic layer at its surface, the receptor may be directly covalently bound to the surface using known linker reagents. Alternatively, an intermediate ligand, such as an antibody, which binds the receptor may first be covalently bound to the surface before this bound intermediate ligand is exposed to the analyte-specific receptor to bind this to the surface. It will be appreciated that covalent and/or affinity bonding may be effective in binding the receptor to the surface. However, when the receptor is bound to the surface, it is important that its ability to bind the analyte species should remain unchanged.

The term antibody as used herein is to be interpreted broadly. Thus, in addition to a whole antibody, the antibody may be a fragment thereof, such as an Fab fragment, an Fv fragment, a single chain fragment (scFv), a

single heavy chain or even a peptide (based on the nucleotide sequence of the antibody gene) having binding activity. The antibodies which may be used in the invention may be obtained by conventional methods and are many times commercially available. Although polyclonal antibodies may be used in the method of the invention, monoclonal antibodies are preferred for their specificity which enhances the accuracy of the method.

Basically, any analyte species present in milk may be analyzed by the method of the invention. Exemplary analyte species are drug residues, such as antibiotics and chemotherapeutics, but also hormones, viruses and toxins.

The measurement of the change in refractive index at the surface may be determined by reflection-optical methods, including both internal and external reflection techniques. Advantageously, the measurement is based on evanescent wave sensing, such as surface plasmon resonance (SPR) detection, Brewster angle refractometry, critical angle refractometry, frustrated total reflection (FTR), evanescent wave ellipsometry, scattered total internal reflection (STIR), optical wave guide sensors, evanescent wave based imaging, such as critical angle resolved imaging, Brewster angle resolved imaging, SPR angle resolved imaging, etc. In the currently preferred method for carrying out the invention, measurement is based on surface plasmon resonance. This technique is described, inter alia, in EP-A-0305109, EP-A-0267142 and WO-A-90/05295. The optical surface which is used in the measurement based on surface plasmon resonance preferably comprises a gold film and a hydrogel bound to the gold film, as described in WO 90/05303. This type of optical surface may easily be regenerated so that a single surface may be used for many analyses. The overall cost per analysis can therefore be reduced considerably. Suitable apparatus incorporating such an optical surface is the BIAcore® system available from Pharmacia Biosensor AB, (Uppsala, Sweden) the methods of operation of which are described in the BIAcore® Methods Manual (Pharmacia

Biosensor AB). In the BIAcore® system, a microfluidic system passes the sample over a sensor chip supporting a gold layer which typically has a thickness of 50 nm. A carboxylated dextran is bound to the gold layer via a linker layer. To this dextran layer analyte species or analyte species receptor may be bound depending on the assay format used.

As apparent from the above, the method of the invention is conveniently performed directly on a raw milk sample. In the case of an inhibition type assay, for the determination of, for example, an antibiotic in the milk, the milk sample is treated with excess anti-antibiotic antibody and then passed over the optical sensor surface which has the antibiotic or an analogue thereto immobilized thereto.

The invention will now be described with reference to the following example and the accompanying drawing, wherein

Fig. 1 is a diagram showing standard curves (relative response vs concentration) for raw and skim milk analyses of sulfamethazine.

Fig. 2 is a graph showing typical relative responses for a number of tanker milk samples.

The analyses were carried out on a BIAcore® system (Pharmacia Biosensor AB, Uppsala, Sweden) with Sensor Chip CM5 as the optical sensor surface.

EXAMPLE 1

Preparation of sensor surface

Sulfamethazine (SMZ) (Mw=278) (No. S-5637, Sigma), 2 mg/ml, was dissolved in 10 mM HCl with 10% dimethylformamide (DMF), pH 3.0, and the solution was filtered through a 0.45 µm filter. The sensor surface was activated for 18 min with 25 µl N-hydroxysuccinimide (NHS)/N-ethyl-N'-(dimethylaminopropyl)carbodiimide (EDC) 1:1, and sulfamethazine was then immobilized to the surface by amine coupling by contacting 30 µl of sulfamethazine solution with the activated surface for 2.5 h. After washing the surface with HBS, any remaining active carboxyl groups were deactivated by placing 25 µl of ethanolamine in

contact with the surface for 18 min, followed by a final wash step. The prepared surface was then conditioned for 1 min with 25 µl 100 mM NaOH + 20% DMF and then for 1 min with 25 µl 100 mM HCl + 20% DMF. The surface was washed three times with 30 µl HBS buffer between each step. The sensor chip was put on a wet paper towel and covered by a box to prevent the chip from drying. Contacting of the sensor surface with the above-mentioned solutions was performed by carefully pipetting the respective solutions onto the surface from a corner thereof and were gently thrown off after incubation.

Analysis of milk samples

Analyses were performed on samples of (i) non-treated, full fat raw milk (i.e. milk taken directly from the milk tank) and (ii) skim milk. To remove the milk fat from raw milk to obtain skim milk, raw milk was centrifuged at 1000 g for 10 min and the fat on top was discarded.

Polyclonal antibodies, prepared from serum from sulfamethazine immunized rabbit by ammonium sulphate saturation and dialysis against PBS with 0.15 M NaCl, were diluted in HBS to a concentration of 4.5 nM. The raw milk and skim milk samples, respectively, were then incubated with an equal volume of antibodies in HBS for 0.5 h at room temperature. The final concentration of antibodies in the milk samples was 2.25 nM.

35 µl samples of milk/antibody from the above prepared raw and skim milk samples were analysed in the BIAcore® system, using a flow rate of 5 µl/min. Regeneration of the surface between the different analyses was performed with 15 µl of 50 mM NaOH, pH 12.2, and 15 µl of 75 mM HCl.

For the preparation of standard curves, standard solutions of sulfamethazine were used to prepare known concentrations of sulfametahzine in milk and HBS buffer. 10 mg of sulfamethazine were dissolved in 10 ml of 50 mM borate buffer, pH 8.5. A working solution was diluted in HBS and finally 100 times in milk (10 µl sulfamethazine + 990 µl milk). This milk was then incubated with antibodies

(in HBS), milk:antibody = 1:1. The final concentration of antibodies in the milk was 2.25 nm.

The relative response for milk from a negative milk sample (no sulfamethazine) with 2.25 nm antibodies varied between 800 and 1100 resonance units (RU), probably depending on the amount of immobilized sulfamethazine and the condition of the sensor chip.

Standard curves of sulfamethazine (SMZ) in skim milk and full fat raw milk are shown in Fig. 1. The relative response is expressed in percent of the average response of negative control samples (i.e. with no sulfamethazine) and the error bars indicate three mean standard deviations (n=10). "A" is negative control (skim milk), "B" is full fat raw milk, and "C" is skim milk.

As can be seen from Fig. 1, the differences between raw milk and skim milk were insignificant, indicating that removal of milk fat has no influence on the results, or , in other words, that milk fat did not interfere in the assay.

Sulfamethazine in milk from a cow treated after morning milk on the 27th of April 1994 with 33 g of the drug sulfamethazine (SMZ) was tested for residues of the drug by HPLC for a number of days after the treatment. At the same time as samples were taken for HPLC, samples of the milk were frozen and tested later by BIAcore® analysis as described above after thawing and defatting. The results are presented in Table 1 below.

TABLE 1

Date of sample	HPLC (ppb SMZ)	BIAcore® (ppb SMZ)
25/4	0	0
26/4	0	0
27/4	10500	14706
28/4	348	588
29/4	21	30
30/4	8	11
1/5	3	3
2/5	1	3
3/5	0	1
4/5	0	0
5/5	0	0

EXAMPLE 2

Tanker milk samples were randomly collected from a screening survey for antimicrobial residues within an integrated control system in Schleswig-Holstein, Germany. All samples were negative in the microbial inhibitor assays (brilliant-black reduction test, a modified Blue Star test and Delvotest SP Special). The samples were frozen and then analysed for sulfamethazine (SMZ) by the BIAcore® analytical procedure described in Example 1 above.

In total 330 tanker milk samples were screened for SMZ residues. 5 of the samples were found to contain SMZ concentrations below 0.9 ppb. In addition, one sample contained between 0.9 and 2.1 ppb SMZ. These samples found positive for SMZ were refrozen and subsequently tested by HPLC for confirmation. In the HPLC confirmation, the sample containing between 0.9 and 2.1 ppb SMZ showed a distinct peak at the retention time for SMZ. The concentration was, however, below the quantification limit (10 ppb) of the HPLC method.

Typical responses in the above analysis of tanker milk samples are shown in Fig. 2. These tanker milk samples are all negative and designated A in the graph. Included in the graph are also two positive standard milk samples (1.4 ppb

SMZ), designated D; two negative standard milk samples, designated C; as well as one incurred tanker milk sample, designated B.

5

CLAIMS

1. A method of determining the presence and/or amount of an analyte species in a raw milk sample, characterized by contacting the raw milk sample, without any preparation thereof for removal of milk constituents, with an optical sensor surface and determining the presence and/or amount of said analyte species by measuring a change in refractive index at the surface caused by a specific interaction at the surface related to the presence of said species in the raw milk sample.

2. The method according to claim 1, characterized in that said analyte species or an analogue thereto is bound to the optical sensor surface and that before the sample is contacted with the optical sensor surface, a ligand capable of reacting with the species, such as an antibody for the analyte species, is added to the raw milk sample.

3. The method according to claim 1, characterized in that a receptor, such as an antibody, for the analyte species, is bound to the sensor surface.

4. The method according to claim 3, characterized in that the receptor is a monoclonal antibody.

5. The method according to any one of claims 1 to 4, characterized in that said analyte species is selected from drugs, hormones, viruses and toxins.

6. The method according to claim 5, characterized in that said drug is an antibiotic or a chemotheraputic.

7. The method according to any one of claims 1 to 6, characterized in that said measurement of the change of refractive index is based on internal reflection.

8. The method according to claim 7, characterized in that said measurement of the change of refractive index is based on surface plasmon resonance.

9. The method according to any one of claims 1 to 8, characterized in that said optical sensor surface comprises a gold film and a hydrogel bound to the gold film.

10. The method according to any one of claims 1 to 9, characterized in that the sample is contacted with the optical sensor surface by passing the sample over the sensor surface in a liquid flow.

11. The method according to any one of claims 1 to 10, characterized by contacting each of a plurality of raw milk samples with a respective one of a corresponding plurality of sensor surfaces and determining the presence and/or amount of said analyte species in each sample.

12. The method according to any one of claims 1 to 11 for determining the presence and/or amount of two or more analyte species in raw milk samples, characterized by contacting each sample with two or more sensor surfaces, each surface being adapted for the meaurement of a respective one of said two or more analyte species.

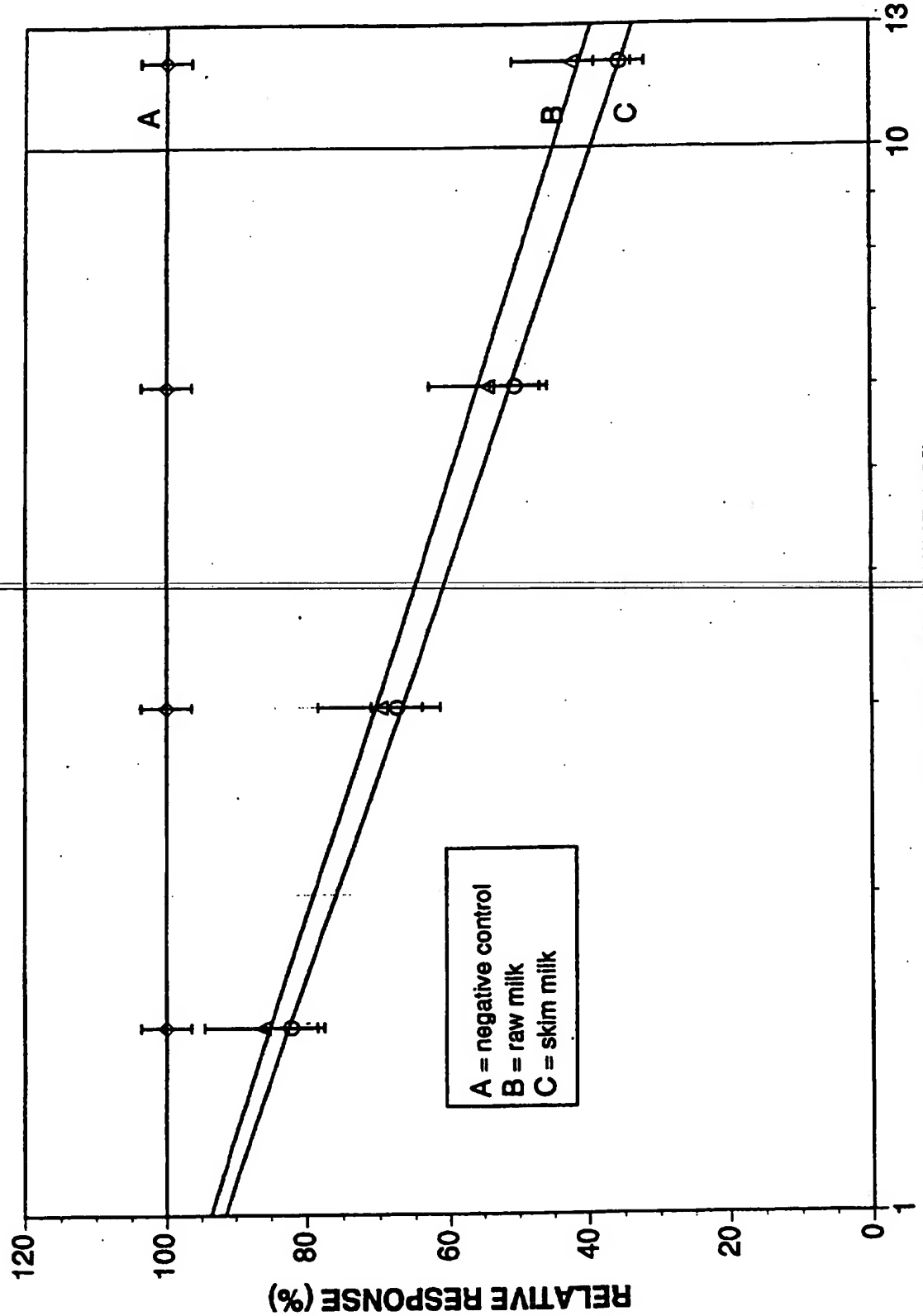
A
B
C
A = negative control
B = raw milk
C = skim milk
RELATIVE RESPONSE (%)
120
100
80
60
40
20
0
SULPHAMETHAZINE (nM)
1
10
13

FIG. 1

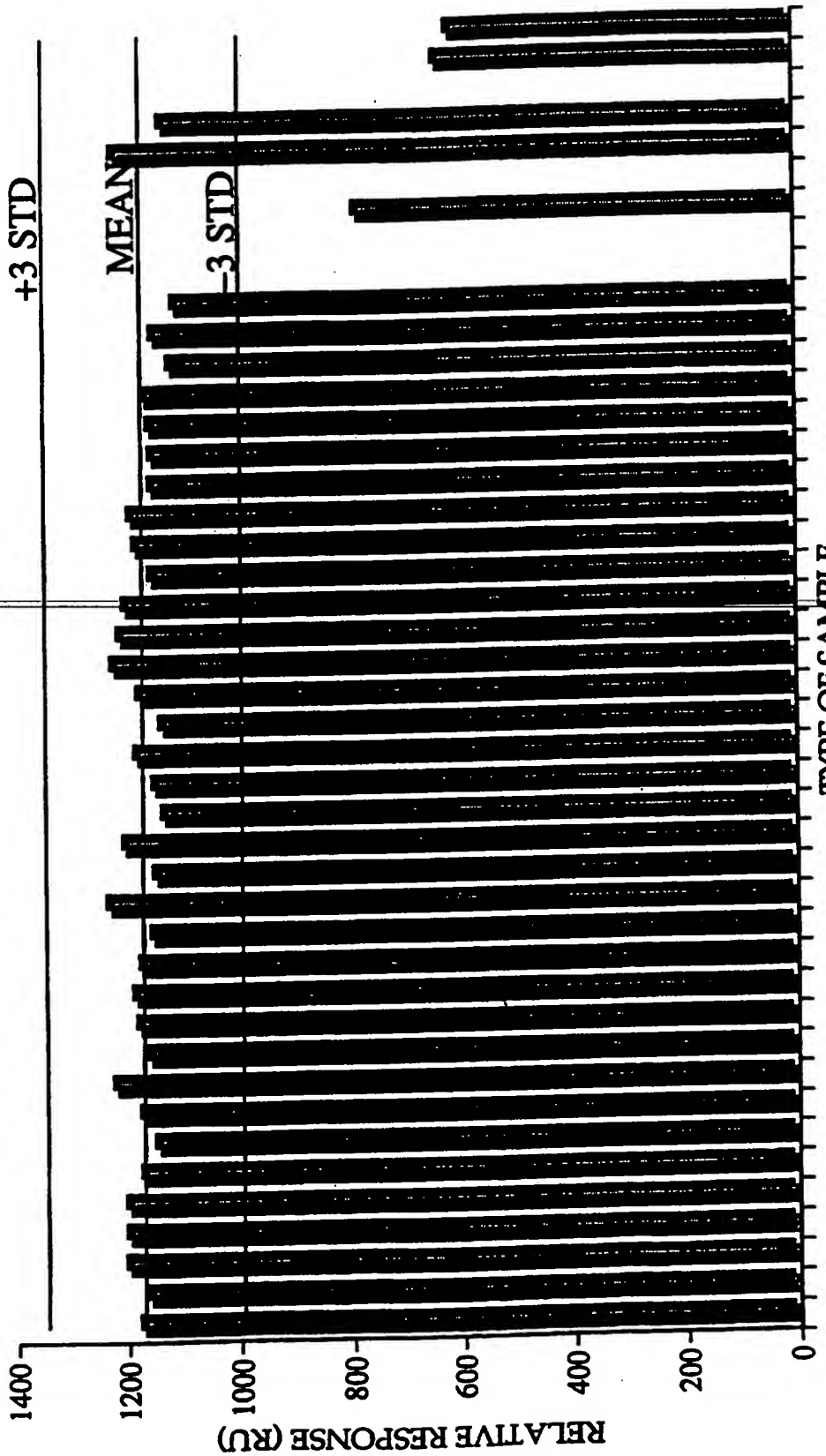
+3 STD
MEAN
-3 STD
RELATIVE RESPONSE (RU)
1400
1200
1000
800
600
400
200
0
TYPE OF SAMPLE
A
B
C
D

FIG. 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 95/01048

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: G01N 33/04, G01N 21/41
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9216838 A1 (GEC-MARCONI LIMITED), 1 October 1992 (01.10.92), page 3, line 13 - line 15; page 10, line 8 - line 10; page 11, line 26 - line 28	1-12
X	EP 0389446 A2 (SOTELCO S.R.L.), 26 Sept 1990 (26.09.90), column 1, line 1 - line 3; column 1, line 42 - line 50; column 2, line 46 - line 48	1-12

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
14 December 1995	09 -01- 1996
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 95/01048

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 9216838	01/10/92	EP-A- 0640216	01/03/95
		GB-A,B- 2255637	11/11/92
		JP-T- 6506058	07/07/94
EP-A2- 0389446	26/09/90	NONE	